Supplementary Information

Differences in peptide physical properties between SCX–RP and (SCX)–RP–RP

Beside overall performance, there were also slight differences in the physical properties among the peptides identified in the two experiments. Although SCX generally excludes highly positive peptides, such as those containing many lysine and arginine residues, due to their late elution from the column [1, 2], we did not expect such a discrepancy in our comparison because the starting sample for both experiments was obtained from the early SCX elution and should have contained mostly peptides possessing two positive charges in solution (e.g., one positive charge at an arginine, lysine, or histidine residue plus one at the terminal amine). There was, however, a minor, but significant, difference of ca. 100 Da in the average molecular weights of the peptide sequences obtained using SCX–RP and RP–RP ($\bar{x}$ = 1433.1 and 1539.0 Da, respectively; Mann-Whitney U test, $p < 0.0001$), correlating with the shorter average sequence length in SCX–RP ($\bar{x}$ = 13.46 and 14.21 residues, respectively; $p < 0.0001$). A shorter sequence length when using SCX–RP has been observed previously by Gilar et al. [3], who hypothesized that the difference was due to a loss of hydrophobic peptides in the first-dimension SCX. Such a hypothesis is inconsistent with our observations; therefore, initially we suspected instead that RP–RP provides higher resolving power, allowing better separation of longer peptides in certain crowded parts of the RP gradients. We observed, however, no significant difference in the peptide theoretical hydrophobicities ($\bar{x}$ = 26.63 and 26.90, respectively; Mann-Whitney U test, $p = 0.206$) that could explain this discrepancy. Instead, we found a small, but significant, difference in the values of pI of the peptides identified ($\bar{x}$ = 5.29 vs. 5.00; Mann-Whitney U test, $p < 0.0001$) in the two experiments, despite their same sample origin; this difference correlated to variations in the number of aspartic acid or glutamic acid residues in the identified sequences (1.97 in SCX–RP vs. 2.50 in RP–RP; $p < 0.0001$). The dissimilarity became more pronounced when we grouped peptides by their presence in either or both experiments: the average number of aspartic acid or glutamic acid residues in peptides unique to the SCX–RP experiment, for peptides found in both experiments, and for peptides unique to RP–RP were 1.66, 2.08, and 2.72, respectively. In contrast, there was virtually no difference between the numbers of positively charged residues (lysine, arginine, histidine) in the peptides used in the two experiments (averages: 1.07 and 1.09, respectively; $p = 0.330$). A high number of these negatively charged residues would be expected

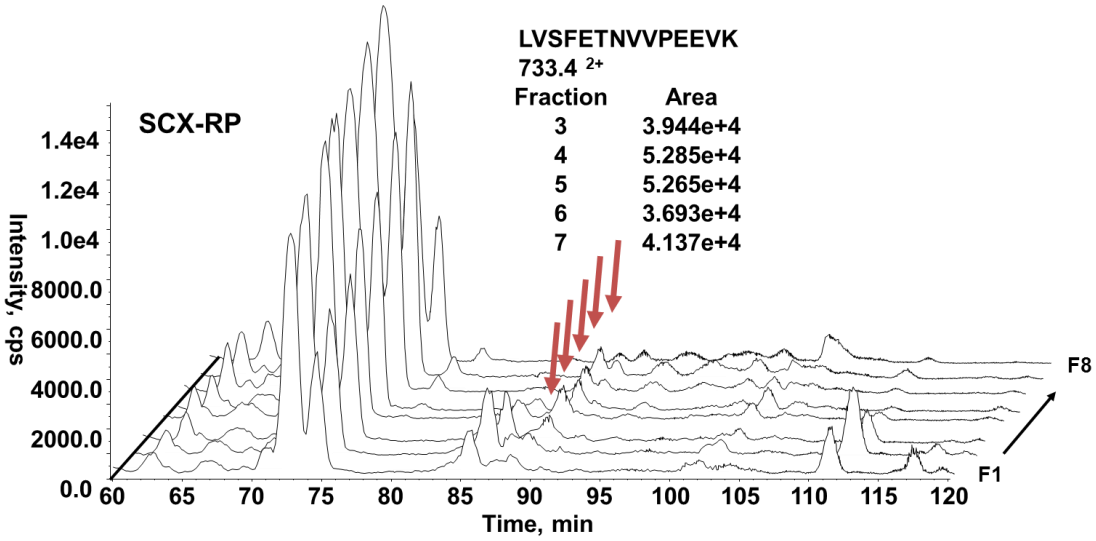
in the early SCX gradients, because they are not retained well on the polysulfoethyl aspartamide surface. Our observation that more negatively charged peptides were identified in RP–RP is intriguing; it might be due to the extra degree of separation improving the probability of ionization of these peptides, regardless of their hydrophobicity.

References

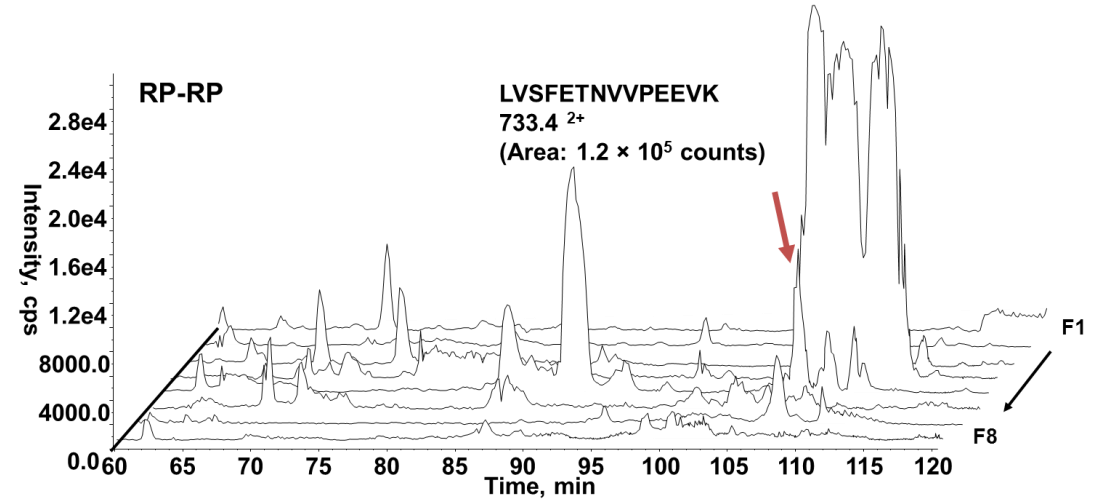
1. K. A. Resing, K. Meyer-Arendt, A. M. Mendoza, L. D. Aveline-Wolf, K. R. Jonscher, K. G. Pierce, W. M. Old, H. T. Cheung, S. Russell, J. L. Wattawa, G. R. Goehle, R. D. Knight and N. G. Ahn, *Analytical Chemistry*, 2004, **76**, 3556–3568.
2. J. Peng, J. E. Elias, C. C. Thoreen, L. J. Licklider and S. P. Gygi, *Journal of Proteome Research*, 2002, **2**, 43–50.
3. M. Gilar, P. Olivova, A. B. Chakraborty, A. Jaworski, S. J. Geromanos and J. C. Gebler, *Electrophoresis*, 2009, **30**, 1157–1167.

Supp. Fig. 1 Dilution effect of broader first-dimension peaks contribute to non-detection of proteins in SCX–RP. In total, 1852 peptides were identified at 95% confidence in RP–RP analysis, but not in SCX–RP analysis; in 61 special cases, peptides that were identified at 95% confidence in RP–RP were observed at lower confidence in SCX–RP, directly leading to the non-detection of their proteins. (a, b): Extracted ion chromatograms at *m*/*z* 733.4 in the eight fractions of SCX–RP and RP–RP, respectively, demonstrating the effect of recombining SCX fractions for RP–RP separation in refocusing the peptide LVSFETNVVPEEVK (733.4^{2+}). (c): MS/MS spectrum of the peptide LVSFETNVVPEEVK in RP–RP (upper) and fraction 4 of SCX–RP (bottom). The lower intensity of the product ion scan in SCX–RP likely contributed to the lower identification confidence. The peptide was identified at 99% confidence in RP–RP, but only 89% confidence in SCX–RP, leading to the non-detection of its protein, phosphoribosylformylglycinamidine synthase, in the SCX–RP experiment.

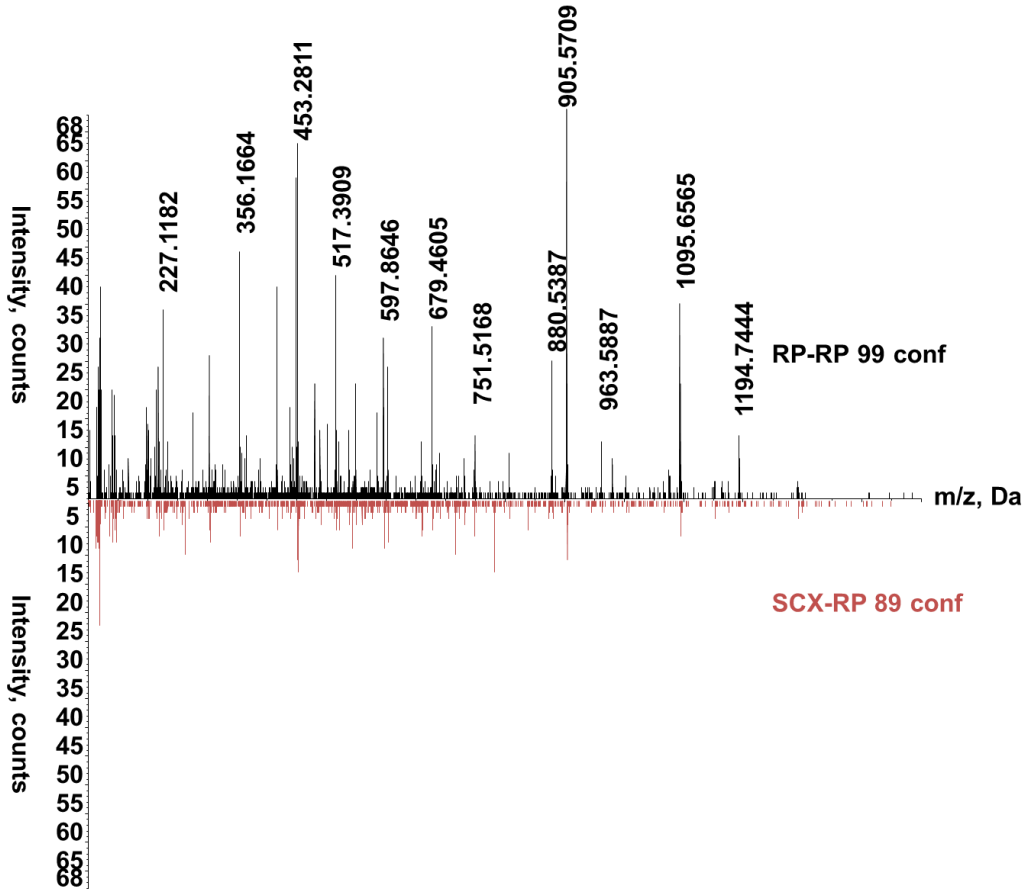
(a)


SCX-RP
LVSFETNVVPEEVK
733.4 2+
Fraction Area
3 3.944e+4
4 5.285e+4
5 5.265e+4
6 3.693e+4
7 4.137e+4
Intensity, cps
1.4e4
1.2e4
1.0e4
8000.0
6000.0
4000.0
2000.0
0.0
60 65 70 75 80 85 90 95 100 105 110 115 120
Time, min
F8
F1

(b)


RP-RP
LVSFETNVVPEEVK
733.4 2+
(Area: 1.2 × 10^5 counts)
Intensity, cps
2.8e4
2.4e4
2.0e4
1.6e4
1.2e4
8000.0
4000.0
0.0
60 65 70 75 80 85 90 95 100 105 110 115 120
Time, min
F1
F8

(c)


227.1182
356.1664
453.2811
517.3909
597.8646
679.4605
751.5168
880.5387
905.5709
963.5887
1095.6565
1194.7444
RP-RP 99 conf
m/z, Da
SCX-RP 89 conf
Intensity, counts
Intensity, counts